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(54) Title: METHOD FOR IN VITRO PRECONDITIONING OF MYOBLASTS BEFORE TRANSPLANTATION

(57) Abstract

A method of pretreating healthy donor's myoblast cultures with growth or trophic factors like basic fibroblast growth factor (bFGF) and with concanavalin A on transplantation to subjects suffering of myopathy like muscular dystrophy is disclosed and claimed. Recipient muscles show a higher percentage of functional cells, a four–fold increase, demonstrated by the higher incidence of dystrophin–positive fibers, and does not require previous preconditioning of recipient muscles by irradiation or toxin administration. The recipient subjects were immunosuppressed with FK 506. When growing myoblasts with 20 μg/ml concanavalin A or 100 ng/ml TPA for two to four days, migration of donor cells in recipient tissue was increased by 3 – 4 fold. This suggests that, when using primary cultures, metalloproteases are secreted by fibroblasts, resulting in a greater degradation of the extracellular matrix. Both metalloproteases and bFGF appear beneficial for the success of the transplantation. The use of recombinant myoblast expressing metalloproteases is also contemplated.

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TITLE OF THE INVENTION

METHOD FOR IN VITRO PRECONDITIONING OF MYOBLASTS BEFORE TRANSPLANTATION

FIELD OF THE INVENTION

The present invention is a method for preconditioning healthy donor's myoblasts *in vitro* before transplantation thereof in compatible patients suffering of recessive myopathies, particularly of muscular dystrophy. This *in vitro* preconditioning improves the success of the transplantation while not requiring an *in vivo* preconditioning of the patient's muscle by irradiation or by administering muscular toxin.

BACKGROUND OF THE INVENTION

Duchenne muscular dystrophy (DMD) is a progressive disease characterized by the lack of dystrophin under the sarcolemmal membrane[6,19,28,37]. One possible way to introduce dystrophin in the muscle fibers of the patients to limit the degeneration is to transplant myoblasts obtained from normal subjects[30,34,35].

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Several groups have tried myoblast transplantations to DMD patients but poor graft success was observed[17,22,24,38]. Even in experimental myoblast transplantation using mdx mice, an animal model of DMD[10,25,29], large amount of dystrophin-positive fibers were observed only when nude mdx mice were previously irradiated to prevent regeneration of the muscle fibers by host myoblasts[32,43]. High percentage of dystrophin-positive fibers was also observed in mdx mice immunosuppressed with FK 506 and in SCID mice, in both cases muscles were previously damaged by notexin injection and irradiated[23,27]. These results indicate that to obtain successful myoblast transplantation, it is necessary to have not only an immunodeficient mouse or a mouse adequately immunosuppressed but also a host muscle which has been adequately preconditioned. It is, however, impossible in clinical studies to use damaging treatments such as marcaine, notexin and irradiation. If good myoblast transplantation results can be obtained without using such techniques, this would be very helpful for myoblast transplantation in humans.

Recently there has been an increasing interest on the effects of basic fibroblast growth factor (bFGF) and other growth factors on myoblast cultures and myoblast cell lines[1,4,5]. Basic FGF has been

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reported to both stimulate proliferation and inhibit differentiation of skeletal myoblasts in vitro[15,16]. Other growth or trophic factors like insulin growth factor I, transferrin, platelet-derived growth factor, epidermal

5   growth factor, adrenocorticotrophin and macrophage colony-stimulating factor as well as C kinase proteins activators or agonists by which the effect of bFGF is mediated[20] may also have similar or even better effects than bFGF on the success of myoblast transplantation[7].

10   The use of these stimulating properties to enhance the success of transplantation by *in vitro* preconditioning of donor's cells and to replace at least partially the use of previously known methods of *in vivo* preconditioning of recipients' cells has never been

15   suggested before.

Furthermore, it has been recently published by Overall and Sodek (1996) that concanavalin A increased the secretion of metalloproteases by fibroblasts. Since these enzymes are believed to be

20   present in primary myoblasts cultures, and since they may be responsible for the degradation of the extracellular matrix, it would be desirable to precondition the myoblasts in the presence of both a growth factor and an inducer of the production of

25   metalloproteases, to increase the distance of migration

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of the transplanted myoblasts and to increase the number of fused myoblasts expressing muscle functional proteins. An attractive alternative would be to use donor myoblasts wherein a gene expressing a metalloprotease is inserted.

Metalloproteases are enzymes necessary for tumor invasion, for cell migration[45], and for restructuration of extracellular matrix during normal tissue remodelization[46]. Matrilysine and gelatinase A are metalloproteases involved in tissue invasion of a plurality of cancer types[47]. The presence of gelatinase A in its active form has been correlated with the generation of new muscle fibers, during muscle degeneration-regeneration process[48]. It has been shown that the activity of gelatinase A can induce cell migration by cleaving laminin-5, an extracellular matrix component, thereby exposing a pro-migratory kryptic site[49].

From the foregoing, it is really apparent that a compound capable of stimulating the expression of a metalloproteases involved in an extra-cellular restructuration, such as phorbol ester or concanavalin A, would be useful to increase the success of transplantation of myoblasts. Since metalloproteases appear to be secreted in the culture medium, it would

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also be useful to test if metalloproteases such as matrilysine, gelatinase A, or other metalloproteases of the same class, could be injected directly with myoblasts in recipient muscle for the same purpose.

5   *STATEMENT OF THE INVENTION*

The present invention relates to a method of *in vitro* preconditioning of myoblasts harvested from healthy donor's biopsy prior to their transplantation in patients affected by recessive myopathies, particularly
10   by Duchenne muscular dystrophy (DMD). In a DMD animal model (mdx), compatible donor mouse myoblasts were grown in culture with muscular growth or trophic factors, particularly, basic Fibroblast Growth Factor (bFGF), before transplanting them in muscles of mdx mice without
15   any previous damaging treatment. A four fold increase in the percentage of muscle fibers expressing dystrophin, which is indicative of functional muscle cells, was obtained with pretreatment with bFGF. These experimental results are expected to verify in naturally occurring
20   dystrophy or other types of recessive myopathies in animal and human subjects, since the mdx mouse is an animal model wherein muscular dystrophy is naturally occurring.

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Furthermore, culturing the myoblasts in the presence of concanavalin A during two to four days prior to transplantation increases by 3 to 4 fold the distance of migration of the transplanted cells into the recipient tissue. Another inducer of the expression of metalloproteases, phorbol ester has been also used and reproduced the same result as for concanavalin A (increase migration and increased number of fused cells expressing a reporter gene). Recombinant myoblasts expressing metalloproteases also produced the same result.

It is therefore an object of the invention to provide a method wherein cultured myoblasts are transplanted in the presence of a metalloprotease. The production of metalloproteases may be induced during the period of culturing of primary myoblast cultures with or without the preconditioning step in the presence of muscle growth factor. Alternatively, the metalloproteases may be expressed by recombinant myoblasts or injected concurrently with the transplanted myoblasts. Transplantation of cells along with a matrix degrading amount of metalloproteases and transplantation of recombinant cells expressing these enzymes are not limited to myoblasts, but could rather be adapted to any type of transplantated cells.

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In accordance with the present invention is provided a method of increasing the number of transplanting donor's myoblasts which are capable of fusing with the myoblasts of a recipient individual suffering of a myopathies, which comprises the steps of: growing said donor's myoblasts in an appropriate culture medium in the presence of fibroblasts and of an agent inducing an increased secretion of an enzyme involved in extracellular matrix destruction prior to injecting said medium, donor's myoblasts and fibroblasts to said recipient individual.

Alternatively is provided a method, wherein the donor's myoblasts are recombinant myoblasts expressing a gene coding for said enzyme.

Is further provided a method which comprises reproducing one of the above methods, and combining to the inducer agent a growth or trophic factor to increase the multiplication of said healthy myoblasts.

In a specific embodiment, said myopathy is Duchenne muscular dystrophy.

In a preferred embodiment, donor's myoblasts consist of a primary myoblast culture obtained from culturing of an enzymatic cell dispersion of donor's muscle biopsy.

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It has been observed that growing of primary cultures of donor's myoblasts, which contain fibroblasts, in the presence of a growth or trophic factor is an *in vitro* preconditioning step that replaces at least in part an *in vivo* preconditioning of said recipient individual's muscular tissue by irradiation or by administering a muscular toxin.

The growth or trophic factor is selected from the group consisting of basic fibroblast growth factor (bFGF), insulin growth factor I, transferrin, platelet-derived growth factor, epidermal growth factor, adrenocorticotrophin, macrophage colony-stimulating factor, protein kinase C activators, agonists thereof, and combinations thereof.

In a preferred embodiment, the growth or trophic factor is basic fibroblast growth factor (bFGF).

In a more preferred embodiment, the primary myoblast culture is grown in the presence of 100 ng of recombinant human bFGF per milliliter of culture medium for a period of time of about 48 hours before transplantation, whereby a four fold increase of the number of functional muscular cells is obtained.

In still a preferred embodiment, the enzyme involved in the extracellular matrix destruction is a

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metalloprotease such as matrilysine and gelatinase A and the inducer agent is phorbol ester or concanavalin A.

In a most preferred embodiment, the inducing agent is concanavalin A (Con A).

5   In a specific embodiment, growing primary myoblast cultures in the presence of 20 μg/ml of Con A for 48 hours resulted in a 3-4 fold increase of the migration distance of transplanted myoblasts and of fused myoblasts.

10   In the most preferred embodiment, primary myoblast cultures are cultured for two days in the presence of both Con A and bFGF, which would result in a superior transplanting success when compared to each treatment alone.

15   **DESCRIPTION OF THE INVENTION**

Although the present trend on research for the treatment of DMD seems to be towards gene therapy, rather than cell therapy, there is still a great deal of work to be done in animal models before either approach,

20   or a mixture of both approaches will be required for the treatment of inherited myopathies such as DMD[32,34].

No satisfactory level of dystrophin expression was obtained following myoblast transplantation not only in clinical trials but also in

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animal experiments not using irradiation[33] combined with
marcaine or notexin destruction of the muscle[26,27]. These
techniques are, however, too damaging, too invasive or
too risky to be used in clinical trials. Basic FGF has

5   been reported to both stimulate proliferation and
inhibit differentiation of skeletal myoblasts by
suppressing muscle regulatory factors such as MyoD and
myogenin[12,41]. Expression of bFGF has been examined in
regenerating skeletal muscles by immunohistochemistry

10   and in situ hybridization, and found to be up-regulated
compared to non-injured muscles[3,11]. Increased skeletal
muscle mitogens have also been observed in homogenates
of regenerating muscles of mdx mice[3]. There are
increased levels of bFGF in extracellular matrix of mdx

15   skeletal muscles[13], mdx satellite cells associated with
repair[3] and such cells respond more sensitively to
exogenous addition of bFGF[14]. There is a high degree of
homology between bFGF from various species[2] therefore
recombinant human bFGF is active on mouse cells[9]. In the

20   present series of experiments, myoblasts were pretreated
with recombinant human bFGF to increase their
proliferation and to verify whether such treatment which
is less invasive could have beneficial effects on
myoblast transplantation.

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In our experiments, primary myoblast cultures from the same donors were grown with or without bFGF and transplanted simultaneously to both *tibialis anterior* (TA) muscles of the same mice. This seems to be a good model to verify the effect of bFGF because the same primary myoblast cultures, the same grafting conditions and the same immunosuppressive state were used. Comparing both TA muscles, in all treated mdx mice, the percentage of β-galactosidase-positive fibers (this enzyme being a reporter gene) were significantly higher in left TA muscles cultures (with bFGF) than in right TA muscles cultures (without bFGF). In the muscles grafted with myoblasts grown with bFGF, the average percentage of hybrid fibers was 34.4%, with two muscles containing over 40% of donor or hybrid fibers. These are the best results ever reported following myoblast transplantation without notexin or irradiation treatment.

In the present study, myoblasts were incubated with bFGF during 48 hours and about 5 millions of these cells (about 1.75 million myogenic cells) were injected in one TA muscle. The same number of myoblasts not incubated with bFGF was injected in the control contralateral TA muscle. The higher percentage of β-galactosidase/ dystrophin-positive fibers was

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therefore not the consequence of a higher proliferation of the myoblasts in vitro before the transplantations.

Our in vitro results indicate that an incubation during 2 days with bFGF did not significantly modify the total number of cells and the percentage of myogenic nuclei. Basic FGF did, however, significantly inhibit the fusion of myoblasts in vitro. This resulted in a small but significant increase (35%) of the percentage of myoblasts among mononuclear cells. This increase seems too small to account alone for the more than four fold increase of effectiveness of myoblast transplantation produced by bFGF. Recently both Partridge[7] and Karpati's[24] group reported that a high percentage (up to 99% in Partridge's results) of the myoblasts injected in a mouse die within 5 days. This dramatic result does not seem attributable to immunological problems since it was observed following autotransplantation[24] or transplantation in nude mice[7]. In our experiments, although there were slightly more cells surviving three days post-transplantation for the cultures treated with bFGF, the difference did not reach a significant level and does not seem to account alone for the 4 fold beneficial effect observed 30 days post transplantation.

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Basic FGF is thought to regulate myogenesis during muscle development and regeneration *in vivo*[3]. The increase percentage of muscle fibers containing the donor gene produced by the addition of bFGF may seem surprising since bFGF was reported to inhibit differentiation of myoblasts in vitro[1,13]. Basic FGF is, however, one of many growth factors which are liberated following muscle damage[7]. These factors, all together, certainly increase myoblast proliferation and eventually muscle repairs. We have also observed that following a two day incubation with bFGF of primary myoblast cultures, myoblast fusion occurred within a few days after removal of bFGF (data not shown). The inhibition by bFGF on myoblast fusion is therefore not irreversible. Basic FGF is already at an increased level in mdx muscle, therefore it is not surprising that direct intramuscular injection did not increase the fusion of the donor myoblasts with the host fibers. In fact, bFGF injected directly in the muscle probably stimulates the proliferation of the host as well as the donor myoblasts and therefore do not favour the donor myoblasts. On the contrary, preliminary stimulation by bFGF of the donor myoblasts in culture may favour these myoblasts to proliferate more and eventually participate more to muscle regeneration than the host myoblasts.

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Though bFGF stimulates the fibroblasts, which an inconvenience for primary myoblast cultures, incubation of myoblast primary culture during only 48 hours with bFGF did not adversely affect our transplantation

5   results and did on the contrary improve them. If primary myoblast cultures were made fibroblast-free by sub-cloning, it is envisageable to precondition the donors' myoblasts for a longer time and increasing this way the number of cells to be transplanted from a relatively

10   small biopsy.

Although the results obtained following transplantation of myoblasts grown with bFGF are not as good than those obtained using irradiation and notexin[27], these results are nevertheless important because no

15   technique to destroy the muscles was used. The proposed in vitro preconditioning method might therefore be used in complete replacement of such in vivo damaging pretreatment of recipient cells, or at least in partial replacement thereof, which will result in a substantial

20   diminution of undesirable effects. The effects of many growth factors and trophic factors on myoblast culture have been reported, it is possible that other factors such as insulin growth factor I, transferrin, platelet-derived growth factor, epidermal growth factor,

25   adrenocorticotrophin and macrophage colony-stimulating

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factor may also have similar or even better effects than bFGF on the success of myoblast transplantation[7]. Furthermore, since the effect of bFGF is mediated by proteins kinase C, pharmacological agents used to enhance the activity of these enzymes (like phorbol esters) or mimicking the effect thereof (agonists) might also be used for preconditioning myoblasts. Therefore, at least one of these factors can be used alone or in combination with or without bFGF to enhance the success of myoblast transplantation. While the mechanism involved remains speculative, bFGF seems to improve the long term viability, multiplication and fusion of myoblasts. Our results suggest that pretreatment of myoblasts with bFGF may be one procedure that may increase the success of myoblast transplantation in DMD patients.

The present invention will be further described by way of the following Examples and Figures, which purpose is to illustrate this invention rather than to limit its scope.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows cross sections of TA muscle of mdx mice 28 days after injection of the transgenic myoblasts. Pairs of serial sections from 3 different

muscles of three mice are illustrated. Panels a and b
illustrate sections of muscles injected with myoblasts
grown without bFGF. Panels c to f illustrate sections of
muscles injected with myoblasts grown with bFGF. In each

5   pair, one section was stained for β-galactosidase
(panels a, c and e). The other section of the pair was
immunostained for dystrophin (panels b, d and f). The
muscles injected with myoblasts grown in presence of
bFGF contained much more β-galactosidase and dystrophin

10   positive fibers than muscles injected with myoblasts
grown without bFGF. Most muscle fibers expressing
β-galactosidase were dystrophin-positive. In each pair
of panels, the same muscle fibers are identified by the
same numbers. Scale bar is 100 μm.

15   Figure 2 shows the number of muscle fibers
positive for X-Gal counted after an injection of 500 000
donor's cells in one site of the *tibialis anterior* of
recipient mice. Imm 7 neo: expresses neomycin. Imm 7
Matrilysine: expresses neomycin and matrilysine. Tn I

20   βgal: untreated transgenic mouse myoblasts expressing β-
Gal. Tn I βGal + TPA: transgenic mouse myoblasts / β-Gal
treated with phorbol ester.

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EXAMPLE 1

MATERIALS AND METHODS

Myoblast cultures

Primary myoblast cultures were established
from muscle biopsies of newborn transgenic mice[26]. The
founder mouse (TnI Lac Z1/29) was provided by Dr.
Hasting (McGill University, Montreal, Canada) onto the
CD1 background and was reproduced in our laboratory.
This transgenic mouse expresses the β-galactosidase gene
under the control of the promoter of the quail fast
skeletal muscle troponin I gene[16]. Blue muscle fibers are
revealed in these transgenic mice following incubation
with a substrate, 5-brom-4-chlor-3-indolyl-
β-D-galactopyronoside (X-gal) (Boehringer Mannheim
Canada, Laval, Canada). Before starting myoblast
cultures, it was necessary to identify transgenic
newborns by X-gal staining of a small muscle biopsy
because heterozygote transgenic mice were used as
parents. Myogenic cells were released from skeletal
muscle fragments of the transgenic newborns by serial
enzyme treatments. First, a one hour digestion was done
with 600 U/ml collagenase (Sigma, St-Louis, Mo, USA).
This was followed by a 30 minute incubation in Hanck's

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balanced salt solution (HBSS) containing 0.1% w/v
trypsin (Gibco Lab, Grand Island, NY, USA). Satellite
cells were placed in 75 cm^2 culture flasks (Coster,
Cambridge, Ma, USA) in proliferating medium, i.e. 199

5   medium (Gibco Lab.) with 15% fetal bovine serum (Gibco
Lab.), 1% penicillin (10,000 U/ml) and 1% streptomycin
(10,000 U/ml).

Myoblast transplantation

         One day after starting culture, the culture

10   medium of some flasks was replaced by medium containing
100 ng/ml human recombinant bFGF (Sigma). Three days
after starting culture, myoblasts were detached from the
flasks with 0.1% trypsin followed by three suspensions
in HBSS and centrifugations (6500 RPM, 5 minutes). The

15   final cell pellet was diluted in only 40 μl of HBSS.

         Seventeen C57BL/10ScSn mdx/mdx mice (mdx
mice) approximately one month old were used for this
experiment. This work was authorized and supervised by
the Laval University Animal Care Committee and was

20   conducted according to the guidelines set out by the
Canadian Council of Animal Care.

         The mdx mice were divided in three groups.
Six mdx mice of one group were grafted in both tibialis
anterior (TA) muscles: myoblasts grown with bFGF were

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injected in the left TA and myoblasts grown without bFGF were injected in the right TA. Myoblasts grown without bFGF were injected in only the left TA of six other mdx mice. These six mdx mice were then injected intramuscularly four times (after grafting 0, +1, +4 and +6 days) either with 10 µl of bFGF (100 ng/ml, 3 mice) or with 10 µl of HBSS (3 mice). The last five mice were grafted in both TA muscle with normal CD1 mouse myoblasts infected with replication defective retroviral vector LNPOZC7 (gift from Dr C. Cepko, Harvard, Boston, MA) which contains the LacZ gene. The left TA muscles were injected with 4 million myoblasts grown with bFGF, while the right TA muscles were injected with 4 million myoblasts grown without bFGF. Three days after grafting, these 5 mice were sacrificed to detect the number of β-galactosidase positive cells which survived in each TA muscle. The numbers of β-galactosidase positive cells were counted in 8 µm sections obtained at every 160 µm throughout the muscle. The total number of cells counted was multiplied by 20 to obtain an estimate of the number of surviving cells and a correction was made to account for the percentage of unlabelled cells in cultures with and without bFGF.

For the myoblast injection, the mice were anesthetized with 0.05 ml of a solution containing 10

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mg/ml of ketamine and 10 mg/ml xylazine. The skin was opened to expose the TA muscle. The myoblast suspension was taken up into a glass micropipette with 50 µm tip (Drummond Scientific Company, Broomall, Pe, USA). The TA muscle was injected at 10 sites with a total of about 5 million cells. The skin was then closed with fine sutures. FK 506 (Fujisawa Pharmaceutical Co Ltd, Osaka, Japan) was administered at 2.5 mg/kg to immunosuppress the animals. Alternatively, the immunosuppressive treatment can be made by other pharmacological agents like cyclosporin (Sandoz), RS61443 (Syntex) or rapamycin (Wyeth-Ayerst) [42].

Muscle examination

Three or twenty-eight days after myoblast transplantation, the mice were sacrificed by intracardiac perfusion with 0.9% saline under deep anesthesia of 10 mg/ml ketamine and 10 mg/ml xylazine. The TA muscles were taken out and immersed in a 30% sucrose solution at 4°C for 12 hours. The specimens were embedded in OCT (Miles Inc, Elkhart, IN, USA) and frozen in liquid nitrogen. Serial cryostat sections (8 µm) of the muscles were thawed on gelatin coated slides. These sections were fixed in 0.25% glutaraldehyde and stained in 0.4 mM X-gal in a dark box overnight (12 hours) at

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room temperature to detect the muscle fibers containing β-galactosidase. Dystrophin was detected on adjacent cryostat sections by an immunoperoxidase technique with a sheep polyclonal antibody against the 60 KD dystrophin fragment (R27, Genica Co, Boston, Ma, USA) and the peroxidase activity was revealed by a 10 minute incubation with 3,3' diaminobenzidine (DAB, 0.5 mg/ml, Sigma) and hydrogen peroxidase (0.015%).

Desmin staining

The primary cultures were washed with PBS and fixed with 100% methanol at -4°C. They were then washed again 3 times with PBS and incubated 1 hr with a mAb anti-human desmin (Dako, Copenhagen, Denmark) diluted 1/50 with PBS containing 1% blocking serum (i.e. 0.33% rabbit serum, 0.33% horse serum and 0.33 fetal calf serum). They were washed 3 times with PBS with 1% blocking serum and incubated 1 hr with a 1/100 dilution (in PBS with 1% blocking serum) of a rabbit anti-mouse immunoglobulin (Dako). Following 3 washes with PBS, the peroxidase activity was revealed with DAB as for dystrophin immunohistochemistry.

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RESULTS

Myoblasts from muscle biopsies of transgenic mice expressing β-galactosidase under a muscle specific promoter were grown with or without bFGF and injected in mdx muscles not previous irradiated or damaged with notexin. A month later, the animals were sacrificed and the injected muscles were examined for the presence of β-galactosidase and dystrophin. Many positive muscle fibers were observed. In our previous experiments, muscles of mdx mice which did not receive injections of transgenic myoblasts remained completely devoid of β-galactosidase-positive fibers[22]. Therefore all β-galactosidase-positive muscle fibers observed in grafted mdx muscles are resulting from the fusion of some donor myoblasts among themselves (donor's fibers) or with the host myoblasts (hybrid fibers). In serial muscle sections, most of the β-galactosidase-positive muscle fibers were observed to be also dystrophin-positive (Fig. 1). In all biopsied TA muscles, the number of β-galactosidase-positive muscle fibers was counted and expressed as a percentage of the total number of fibers in a cross section. The sections containing of the maximum percentage of β-galactosidase-positive muscle fibers were selected for each muscle. In

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mdx mice grated in both TA muscles, the percentage of β-galactosidase-positive muscle fibers in the left TA muscle (grafted with myoblasts grown with bFGF) was compared with that in the right TA muscle (grafted with

5   myoblasts grown without bFGF) of the same mouse (Table 1). Without notexin and irradiation, only a low percentage of hybrid or donor muscle fibers were observed in the right TA muscle i.e. the mean number of β-galactosidase-positive fibers per muscle cross section

10   was 156.3 giving a mean percentage of β-galactosidase-positive fibers of 8.396. The left TA muscles contained, however, significantly more hybrid or donor muscle fibers, i.e. the mean number of β-galactosidase-positive fibers per muscle cross section was 773.7 thus giving a

15   mean percentage of β-galactosidase-positive fibers equal to 34.4% (Fig. 1). This is more than a four fold increase in the efficacy of myoblast transplantation produced by the addition of bFGF to the culture medium.

We have also investigated whether the
20   beneficial effect of bFGF could be obtained by injecting it directly in the muscle at 4 intervals after myoblast transplantation. No significant difference in the percentage of hybrid or donor muscle fibers (i.e. β-galactosidase positive fibers) was observed between

25   the groups which received intramuscular injections of

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bFGF and those which received HBSS injections (control) (Table 2). The percentage of β-galactosidase positive muscle fibers was, however, higher following repeated injection of HBSS (14.8%) or of bFGF (15.9%) than

5   following injection of myoblasts alone grown without bFGF (Table 1, 8.3%). This may be due to damage produced by the repeated injections which may increase the regeneration process.

It has been reported recently by Huard et

10 al.[21] and by Beauchamp et al.[7], that a high percentage of the myoblasts injected in a muscle died within the first few days following their transplantation. To examine whether the increase efficiency of myoblast transplantation following culture with bFGF could be due

15 to a reduced cell death, we have labelled normal CD1 primary cultures grown with or without bFGF with a retroviral vector containing the β-galactosidase gene under an LTR promoter. Normal myoblasts were labelled with a retroviral expressing β-galactosidase because

20 only mature myoblasts and myotubes of transgenic TnI LacZ 1/29 can express β-galactosidase. With labelling using a retroviral vector a higher percentage of the cells in the primary culture expressed the reporter gene. The retrovirally labelled cells were then injected

25 in a muscle of 5 mice. We examined the number of

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β-galactosidase positive cells 3 days after their transplantation. In all 5 mice, the number of the cells was not significantly higher in left TA muscles (with bFGF) (3.29 ± 1.54 x 10^5 cells) than in right TA muscles

5   (without bFGF 2.13 ± 0.40 x 10^5 cells). Note that since 4 x 10^6 cells were injected in each muscle, there is only 5.3% of the injected cells surviving at 3 days without bFGF while only 8.2% of the injected cells survived with bFGF.

10   To try to understand the beneficial effects of bFGF on myoblast transplantation, we examined the effect of a short stimulation (2 days) with 100 ng/ml bFGF on primary myoblast cultures. The total number of cells in each flask was not significant different (31.9

15   ± 6.8 x 10^6 with bFGF n = 5, 30.0 ± 5.8 x 10^6 without FGF n = 9, unpaired t-test: p = 0.573). The myoblasts and myotubes were then identified by revealing desmin by immunoperoxidase. In these cultures, there was no difference in the percentage of myogenic nuclei (nuclei

20   in myoblasts and in myotubes) between the two groups of cultures (Table 3, line 1). More myogenic cells were however fused in the absence of bFGF (Table 3, line 2). There was an higher percentage of the total nuclei (including myoblasts, myotubes and fibroblasts) which

25   were myoblast nuclei in cultures containing bFGF

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(Table 3, line 3). The increase of myoblasts was more clear when the percentage of myoblasts was calculated among mononuclear cells (excluding the myotubes) (Table 3, lines 4 and 5). This was however only a 35% increase.

Table 1:   Effect of culture with or without bFGF on the formation of muscle fibers containing donor's gene in mdx mice

No of mdx mice	no bFGF (right TA muscle) No (%) of β-gal. positive fibers	with bFGF (left TA muscle) No (%) of β-gal. positive fibers
1	170(11.0)	514(19.3)
2	259(11.9)	438(20.4)
3	259(13.1)	1007(37.4)
4	57(4.1)	695(34.0)
5	139(6.1)	848(43.8)
6	54(3.6)	1140(51.7)
Mean ± SD	156.3±91.5(8.3±4.2)#	773.7±275.8(34.4±12.8)#

#. Paired t-test indicated a significant difference ($p < 0.05$)

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Table 2:   Effects of bFGF on primary myoblast culture

	no bFGF (mean±SD)	with bFGF (mean±SD)	sign
1) % of myoblast and myotube nucleic relative to total nuclei	34.5±5.3	35.1±4.8	0.81
2) % of myotube nuclei relative to total myotube and myoblast nuclei	40.8±8.0	11.5±6.6	0.0001
3) % myoblast nuclei relative to total nuclei	21.1±3.6	30.9±3.8	0.0001
4) % myoblast nuclei relative to non myotube nuclei	23.9±5.4	32.2±4.1	0.001
5) % of non-myoblast nuclei relative to non myotube nuclei	76.1±5.4	67.8±4.1	0.001

Table 3:   Effect of intramuscular injections of bFGF
               in mdx mice

	No (5%) of β-gal. positive fibers	Mean±SD
HBSS IM injections		
1	180(12.4)	
2	421(14.1)	372.0±172.8 (14.8±2.9)
3	515(18.0)	
bFGF IM injections		
1	176(7.4)	
2	482(24.1)	289.7±167.5 (15.9±8.4) T test indicated no significant difference (p>.05)
3	211(16.3)	

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EXAMPLE 2

The above results can be extrapolated to an *in vivo* utility and verified in patients suffering of muscular dystrophy. The healthy donors and DMD recipients should be matched, if possible, upon their compatibility for the MHC (HLA)-class I (A,B,C) and -class II (Dr) antigens. The dystrophic patients should undertake an immunosuppressive treatment by being administered, for example, FK 506, cyclosporin, RS61443 or rapamycin. Donors' biopsy would then be treated substantially in accordance with the procedures given in Example 1 with regard to mice myoblasts. The success of the transplantation might be monitored by measuring the incidence of dystrophin-positive fibers from a biopsy obtained from the site of transplantation and by evaluating the resulting increase of muscular strength[39].

EXAMPLE 3

Myoblasts infected with a retrovirus expressing a beta-galactosidase gene have been cultured for four days in the presence or absence of 20μg/ml concanavalin A, a lectine which stimulates the expression of metalloproteases. These myoblasts were then injected in one single site of the anterior tibialis muscle of eight mice, in order to verify the

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degree with which the transplanted cells are capable of migrating through the recipient tissue. After thirty days, mice were sacrificed and the muscle tissue was harvested, frozen and 10μm thick slices were mounted on slides. The presence of beta-galactosidase was revealed with X-Gal. Labelled cells were observed at a distance which is 3 to 4 fold greater in the mice muscle treated with concanavalin A. Concanavalin A is known to induce the secretion of metalloproteases by the fibroblasts present in the primary cultures. Therefore, the presence of metalloproteases in the preconditioning medium or during the transplantation is beneficial to the spreading of the transplanted cells from the site of the injection through the recipient muscle tissue.

Three experiments confirmed that an increased expression of enzymes involved in the destruction of the extracellular matrix indeed increases the migration of myoblasts.

First experiment: Myoblasts obtained from CMVLacZ mice (expressing a Beta-galactosidase gene) were cultured for two (2) days in the presence of twenty (20) μg/ml concanavalin A, a lectine which stimulates the expression of metalloproteases. When these myoblasts were injected in the anterior tibialis muscle of recipient mice, the migration of transplanted cells was

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increased by three (3) fold comparatively to myoblasts having not received the concanavalin treatment.

     <u>Second experiment:</u> primary cultures of myoblasts were obtained from transgenic mice (TnI-βGal + TPA). Myoblasts were cultured for two (2) days in the presence of phorbol ester (TPA 100 ng/ml) which also stimulates the expression metalloproteases. These treated myoblasts generated four times more fibers on an area four fold greater than for transplanted myoblasts injected from a non-treated preparation (See figure 2; n = 5 mice).

     <u>Third experiment:</u> To verify if metalloproteases are really involved in the increased spread of transplanted myoblasts, we have stably transfected an immortalized myoblast cell line already expressing a β-Gal gene with an expression vector comprising human matrilysine gene and a neomycine resistance gene[50]. This transfection greatly increased the fusion capacity *in vitro* and the generation of fibers *in vivo*. Expression of β-Gal gene was measured. The clone Imm 7 Matrilysine was compared to the same clone (Imm7) transfected solely with the neomycine resistance gene. As seen from figure 2, the injection of 500,000 cells in the anterior tibialis muscle of recipient mice (non irradiated and non injected with a

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myotoxic agent such as notexin) resulted in the average presence (n = 8 mice) of 39 positive fibers for beta-galactosidase following the injection of the clone expressing matrilysine, while no fiber has been observed

5   with the control clone, three (3) weeks after the injection.

Fourth experiment: Tumoral myoblasts obtained from G8 mice transfected with the same construct (matrilysine and neomycine recombinant) and

10   labelled with a fluorescent die (PKH26) have been allowed to migrate for eight (8) days following injection in the anterior tibialis muscle of recipient mice. The distance of migration was equivalent to three to four times the distance observed with the control

15   construct.

When myoblasts are cloned, which results in the removal of fibroblasts, it is believed that recombinant myoblasts expressing a metalloprotease gene product could be useful in increasing further the

20   success of transplantation per se or along with the increase due to the preconditioning step with a growth factor.

Alternatively, metalloproteases could be directly injected with the donor's myoblasts to increase

25   the migration of the transplanted cells.

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    Although the present invention has been
described hereinabove by way of preferred embodiments
thereof, it can be modified, without departing from the
spirit and nature of the subject invention as defined in
5   the appended claims.

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